EXPERIMENTAL STUDY

Regulation of nuclear receptor and cofactor expression in breast cancer cell lines

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Abstract

Objective: The aim of this study was to compare the expression profile of nuclear receptors (NRs) and cofactors in different breast cancer cell lines as well as their regulation by estradiol, insulin and progesterone R5020.

Methods: Expression of NRs and cofactors were determined from MCF-7, T-47D and ZR-75-1 breast cancer cell lines. Multiprobe ribonuclease protection assay and real-time RT-PCR were used to quantify mRNA levels of steroid receptors, vitamin D receptors (VDR) and retinoic acid receptors (RAR) and cofactors: amplified in breast cancer-1, cyclic AMP response element binding protein (CBP), p300/CBP-associated factor, p300, nuclear receptor corepressor and silencing mediator of repressed transcription.

Results: Basal expression levels of NRs and cofactors varied depending on the cell line. Cell line-specific regulation of androgen receptor, estrogen receptor- α (ER α), RAR α , RAR γ and VDR expression was observed after estradiol treatment. Likewise, differences in the regulation of ER α , RAR α and VDR expression after R5020 treatment were observed. We did not observe significant regulation of cofactor expression after estradiol, insulin or progesterone treatment in any cell line analyzed.

Conclusions: The results showed that not only is the expression profile of the NRs and cofactors cell line specific but also the regulation of NR expression. Thus the determinants of the ligand action (receptor and cofactor expression) varied considerably among different cell clones of the breast cancer cells. This suggested a gradient of NR-ligand sensitivities in the hormone-dependent breast cancers, which produces an additional challenge in developing novel ligands for hormone replacement therapy and breast cancer treatment.

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Introduction

Nuclear receptors (NRs) are a large family of transcription factors that regulate development, homeostasis, proliferation and differentiation (1). Ligands of NRs include steroid hormones, retinoids, vitamin D, fatty acids and prostaglandins. Non-liganded receptors such as thyroid receptor and retinoic acid receptor (RAR) can repress basal transcription through the binding of the corepressors, nuclear receptor corepressor (N-CoR) and silencing mediator of repressed transcription (SMRT) (2, 3). Upon ligand binding the conformation of the NR is changed and corepressors are dissociated. Ligand-bound receptors form either homo- or heterodimers and bind to specific response elements in DNA. A receptor dimer associates with the general transcription machinery either directly or via cofactors, and

activates or represses transcription. Several cofactors have been shown to associate with NRs during the transactivation process. These include p160 coactivators: steroid receptor coactivator-1 (SRC-1) (4), transcription intermediary factor-2 (TIF2) (5) and amplified in breast cancer-1 (AIB1, also known as RAC3, ACTR, TRAM) (6–9) as well as coactivators that have strong intrinsic histone acetyltransferase activity, such as p300 (10), cyclic AMP response element binding protein (CBP) (10, 11) and p300/CBP-associated factor (pCAF) (12). It has been proposed that histone acetylation derepresses the chromatin and thus allows the binding and assembly of the transcription machinery.

Although the spatio-temporal expression pattern of steroid receptor determines whether the cognate steroid has a physiological role in the tissue, there are several

other factors contributing to this response as well. Several NRs, for example progesterone receptor (PR) and RARs, are expressed as two or more isoforms, which are transcribed from different promoter regions of the same gene or are splicing variants of the primary transcript (13–18). Results from transfection assays show that the two isoforms of human PR have partly different transcriptional activities (19–21) which has been suggested to be due to different cofactor binding (22). Agonists and antagonists induce different conformational changes in the receptor determining cofactor binding to the receptor, as shown for estrogen receptor- α (ER α) (23, 24). The conformation of the ligand–receptor complex is affected upon binding to the DNA response elements and this also affects the recruitment of coactivators, as shown for ER α and ER β (25–28). Expression levels of cofactors like N-CoR, SMRT, SRC-1 and ribosomal protein L7 affect whether the anti-estrogen tamoxifen acts as an agonist or an antagonist (29–32). So the physiological response of the ligand depends on the tissue distribution of the receptor and its dimerizing partners and on the spatio-temporally regulated expression of coactivators and corepressors. In addition, phosphorylation of the unliganded receptor may affect cofactor binding and thus the activity of the receptor. For example, phosphorylation of activation function 1 of ER β by mitogen-activated protein kinase modulates the interactions of unliganded ER β with the cofactor SRC-1 (33).

Estrogens and progestins are widely used in contraception and in hormone replacement therapy (HRT). The growth-stimulatory effects of estrogens on normal mammary epithelium and breast cancer cells are well known. Progestins can induce either proliferation or differentiation of mammary epithelial cells. A cross-talk between PR and growth factor and cytokine-signaling pathways has been suggested to explain the complexity (34). At the molecular level, estrogens up-regulate the expression of PR in breast cancer cells (35, 36). However, the effect of estrogen on its own receptors depends on the breast cancer cell line. In MCF-7 cells, the levels of ER α are decreased and in T-47D and ZR-75-1 cells increased by estrogen stimulation (37–39). The expression of ER β is increased by estrogen in T-47D cells (40). Progestins down-regulate PR, ER α (39, 41, 42) and ER β (43) in breast cancer cell lines. The effect of estrogens and progestins on other NRs is much less studied, although androgen receptor (AR), glucocorticoid receptor (GR), RAR, retinoic X receptor (RXR) and vitamin D receptor (VDR) are commonly found in breast carcinomas and cell lines. The expression levels of NRs might be regulated during carcinogenesis since ER α was shown to be up-regulated (44) and ER β down-regulated during carcinogenesis of breast tumors (44, 45). Furthermore, the expression of RAR β has been found to be decreased during breast carcinogenesis (46). Lawrence *et al.* (47) linked overexpression of RXR with an

increased risk for the development of invasive breast cancer.

Several studies have suggested that changes in the expression levels of cofactors might also contribute to carcinogenesis or to the hormonal response of the tumor. Overexpression of coactivators AIB1 and steroid receptor RNA activator has been shown in primary breast tumors, suggesting that factors enhancing ER activity are up-regulated in breast tumors (6, 48). Also, the expression levels of TIF2 and CBP were increased in intraductal carcinomas compared with normal mammary gland (49). In the same study, it was shown that the expression level of N-CoR mRNA decreases during the development of invasive ductal carcinomas from intraductal carcinomas.

Although there are several studies that have determined the expression levels of NRs or cofactors in breast cancer, further knowledge of the regulation of NR and cofactor expression is needed for a better understanding of the response of hormonal treatments in breast cancer and for development of new treatments. We wanted to compare the three mainly studied breast cancer cell lines, MCF-7, T-47D and ZR-75-1, and analyze the expression of different NRs and cofactors as well as their regulation by estradiol, insulin and progestin so that the results are comparable between these cell lines.

Materials and methods

Cell culture

Human breast cancer cell lines, MCF-7, T-47D and ZR-75-1, were cultured in Dulbecco's modified Eagles' medium (DMEM)/F12 medium (Gibco-BRL, Grand Island, NY, USA) containing 5% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μ g/ml) and bovine insulin (10 ng/ml; Sigma, St Louis, MO, USA). MCF-7 cells were supplemented with 1 nmol/l 17 β -estradiol (Sigma). One week before the experiment, breast cancer cells were transferred to phenol red-free DMEM/F12 medium containing antibiotics and 5% dextran charcoal-treated serum. Medium was changed every second day. When the short-term effects of insulin and estradiol were studied the medium contained no other supplements. Cells were washed with phosphate-buffered saline and trypsinized 24 h after the addition of insulin (1 μ g/ml), estradiol (1 nmol/l) or a combination of both. When the effects of long-term estradiol treatment were studied, the medium contained estradiol (1 nmol/l) and insulin (1 μ g/ml) for 1 week. When the effects of R5020 (Schering Aktiengesellschaft, Berlin, Germany) treatment were studied, the medium also contained estradiol (1 nmol/l) and insulin (1 μ g/ml) for 1 week and cells were harvested 24 h after the addition of R5020 (10 nmol/l). Total RNA was isolated using TRIzol reagent (Gibco-BRL). Human promyelocytic cell line HL-60 was cultured in

RPMI medium (Gibco-BRL) containing 10% foetal bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml), HEPES (10 mmol/l) and glutamine (2 mmol/l). HL-60 cells were treated for 48 h with 200 mmol/l 8CTP-cAMP (Sigma) and 1 mmol/l SR11237 (gift from H Gronemeyer). COS cells were grown in DMEM/F12 medium containing 5% fetal bovine serum, penicillin and streptomycin. COS cells were transfected using the lipofectamine reagent (Gibco-BRL) according to the supplier's instructions.

Ribonuclease protection assay (RPA)

Three sets of RPA probes were designed (Table 1). Steroid receptor set (SR set) consisted of probes for detecting GR, AR, PR, ERβ, cyclin A, VDR and ERα mRNAs. The VDR set comprised probes for detecting VDR, RARα, RARβ, RARγ, RXRα, RXRβ and RXRγ mRNAs and the cofactor set probes for N-CoR, SMRT, pCAF, CBP, TIF2, AIB1, SRC-1 and p300. The probes of the SR set were done by TA cloning the selected PCR fragment amplified from each gene's cDNA to transcription vector pGEM-T Easy (Promega, Madison, WI, USA). The probes of VDR and cofactor sets were done by subcloning the selected PCR fragment to pSG5 vector (Stratagene, La Jolla, CA, USA) using BamHI and EcoRI digestion sites, which were included in the PCR primers. The orientation and the sequence of the constructs were confirmed by DNA sequencing. The antisense [³²P] uridine 5'-triphosphate labeled probes were transcribed from linearized plasmids by

using the RiboQuant *in vitro* transcription kit (Pharmingen, San Diego, CA, USA). The relative amounts of the probes in the SR set were: GR, PR, ERβ, cyclin A and VDR 10%, AR 24% and ERα 18%. Likewise, the relative amounts in the VDR set were: VDR and RXRβ 14%, RXRα, RXRγ and RARγ 10%, RARα and RARβ 21%. In the cofactor set, the relative probe amounts were: SMRT, TIF2, AIB1, SRC-1 and p300 16%, N-CoR and CBP 8% and pCAF 4%. ³²P-labeled ribosomal 18S probe was synthesized from plasmid pTRI RNA 18S (Ambion, Austin, TX, USA) by using MEGashortscript T7 kit (Ambion). Total RNA (5 µg) from each sample was hybridized with a 500 000 c.p.m. ³²P-labeled RNA probe set (SR, VDR or cofactor set) (specific activity 1.7 × 10⁹ c.p.m./µg) and with 30 000 c.p.m. ³²P-18S RNA probe (specific activity 89 000 c.p.m./µg) by using an RPA kit (RPA III; Ambion). The length of the synthesized 18S probe was 128 bp and the length of the protected hybridized 18S was 80 bp. Yeast RNA was used as a negative control. In addition, an 8 µg sample of RNA was used to control that probes were in molar excess in hybridization reactions. Hybridization was done at 43 °C for at least 16 h. Hybrids were resolved in 5% polyacrylamide gel containing 8 M urea and visualized by autoradiography. Gels were scanned using PhosphorImager Storm (Molecular Dynamics, Sunnyvale, CA, USA) or films were scanned using a personal densitometer SI (Molecular Dynamics). The intensities of hybrids were calculated with ImageQuant software (version 5.0; Molecular Dynamics). Before experiments, each probe of the VDR and cofactor sets was labeled alone to confirm the specificity. Hybrids were identified based on their expected size. The right hybrid for AR, ERα, ERβ, PR, VDR, RARα, RARβ and RARγ was verified using RNA from COS cells transfected with the corresponding cDNA. In order to demonstrate that the cofactor set identified all the corresponding genes, total RNA (4 µg) from HL-60 cells was hybridized with the ³³P-labeled cofactor set and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe using Pharmingen's RPA kit according to the supplier's instructions. The intensities of 18S hybrids were used for normalization of loading between samples. To assess whether insulin, estradiol and progesterone regulated the expression levels, the relative intensity of the hormone-treated sample was divided by the relative intensity of the control sample. Statistical significance of differences was analyzed using the non-parametric Mann-Whitney U test (SPSS 10.1 for Windows).

Table 1 Probes for RPA analysis.

Gene	AC#	mRNA region	Size ¹	Restriction enzyme ²
GR	M10901	1741–2240	500	Sall
AR	M23263	2641–3100	460	Sall
PR	X51730	3945–4344	400	Sall
ERβ	X99101	503–818	316	Sall
Cyclin A	A32139	598–897	300	Sall
ERα	M12674	1846–2065	220	Sall
VDR	AF026260	1–326	326	Xbal
RXRα	X52773	83–372	290	Xbal
RXRβ	M84820	141–399	259	XhoI
RXRγ	U38480	9–240	232	XhoI
RARγ	M24857	404–605	202	XhoI
RARβ	X07282	333–515	183	XhoI
RARα	X06538	309–474	166	Xbal
N-CoR	AA564930	72–432	361	HindIII
SMRT	U37146	600–910	311	HindIII
pCAF	U57317	901–1166	266	XhoI
CBP	U47741	598–831	234	XhoI
TIF2	X97674	3111–3310	200	XhoI
AIB1	AF010227	2594–2772	179	BamHI
SRC-1e	U19179	1421–1580	160	BamHI
SRC-1a ³	U90661	4053–4197	145	BamHI
p300	U01877	7404–7533	130	BamHI

AC#, GenBank accession number; ¹length of the protected probe – RNA hybrid in base pairs; ²restriction enzyme used to linearize RPA – probe plasmid; ³probe for SRC-1a is the same as for SRC-1e but when hybridized to SRC-1a it yields a shorter hybrid.

Real-time RT-PCR

Relative quantification of ERα and VDR mRNA levels was also done using real-time RT-PCR. Acidic ribosomal phosphoprotein P0 (RPLP0) was used as an endogenous RNA control. Primer pairs were selected in different exons in order to avoid amplification

from possible contaminating genomic DNA. Primers for the reference gene RPLP0 were chosen according to Bièche *et al.* (50). The following primers were used for PCR: ER α forward 5'-CCACTCAACAGCGTGTCTC-3', ER α reverse 5'-GGCAGATTCCATAGCCATAC-3', VDR forward 5'-CTGACCCTGGAGACTTTGAC-3', VDR reverse 5'-TTCCTCTGCACTTCTCATC-3', RPLP0 forward 5'-GGCGACCTGGAAGTCCAAC-3', RPLP0 reverse 5'-CCATCAGCACCACAGCCTTC-3'. One-step RT-PCR was performed using LightCycler RNA Master SYBR Green I kit (Roche Molecular Biochemicals, Mannheim, Germany). Total reaction volume was 20 μ l containing 200 ng total RNA and 0.5 μ M of each primer. Mn(OAc)₂ concentration was 2.5 mM in ER α assays, 3 mM in VDR assays and 3.25 mM in RPLP0 assays. Reactions were performed as described by the manufacturer. Reaction conditions in the LightCycler instrument (Roche) were chosen according to the manufacturer's recommendations. Reverse transcription was performed at 61 °C for 20 min followed by denaturation at 95 °C for 30 s. PCR cycles consisted of three steps: denaturation at 95 °C for 1 s, annealing at 55 °C (ER α and VDR) or 58 °C (RPLP0) for 5 s and elongation at 72 °C for 11 s (ER α), 12 s (VDR) or 7 s (RPLP0). In total, 38 cycles were performed. Fluorescence was measured at the end of each elongation step at 72 °C. However, when amplifying RPLP0 the fluorescence was measured at 81 °C to avoid measurement of fluorescence due to an undesired faintly amplified product, which had a melting temperature lower than 81 °C. The sizes of the amplified products were 242 bp for ER α , 276 bp for VDR and 149 bp for RPLP0. After amplification, the specificity of the PCR products was verified by a melting curve analysis using the LightCycler instrument. Agarose gel electrophoresis was performed to verify the length of the PCR products. The fit points method was used to determine the crossing point at which the fluorescence of each sample rose appreciably above the background fluorescence. Relative quantification of the target gene in comparison with the reference gene RPLP0 was calculated by the following equation (51):

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta C_{\text{Ptarget}}(\text{control-sample})}}{(E_{\text{ref}})^{\Delta C_{\text{Pref}}(\text{control-sample})}}$$

Statistical significance of differences was analyzed using the non-parametric Mann-Whitney U test.

Results

Expression of NRs

Breast cancer cell lines are widely used to model the effects of estrogen, progesterone and their analogs. In this study, the expression levels of different NRs in MCF-7, T-47D and ZR-75-1 cell lines were characterized by using multiprobe RPA analysis in which expression of several mRNAs can be quantitated in

one hybridization reaction. RPA analysis was used to detect the expression of AR, ER α , ER β , GR, PR, VDR, RAR α , RAR β , RAR γ , RXR α , RXR β and RXR γ mRNAs. All cell lines expressed mRNAs for steroid receptors AR, GR, PR, ER α and ER β (Fig. 1). However, the expression levels of control samples varied several fold between the different cell lines (see lanes 1, 7 and 13 in Fig. 1). Differences between the cell lines were also seen in the expression of VDR and RAR γ (see lanes 1, 7 and 13 in Fig. 2). In T-47D cells, VDR was expressed abundantly whereas in the other two cell lines VDR was expressed at low levels. MCF-7 and T-47D cells expressed RAR γ mRNA abundantly, whereas in ZR-75-1 cells it was expressed at moderate levels. RAR α was expressed in all cell lines. All cell lines expressed RXR α but the mRNA level was too low to be quantified accurately. RAR β , RXR β and RXR γ mRNAs were undetectable in the RPA analysis.

Figure 1 Steroid receptor and cyclin A (cycA) mRNA expression levels in MCF-7 (lanes 1–6), T-47D (lanes 7–12) and ZR-75-1 cells (lanes 13–18) were determined using RPA. Control samples (no hormonal treatment) were run in lanes 1, 7 and 13. Cells were treated with 1 μ g/ml insulin for 24 h (lanes 2, 8 and 14) or with 1 nmol/l estradiol for 24 h (lanes 3, 9 and 15) or with a combination of insulin and estradiol for 24 h (lanes 4, 10 and 16) or cells were grown in the presence of insulin and estradiol for 1 week (lanes 5, 11 and 17) and were treated for 24 h with 10 nmol/l R5020 (lanes 6, 12 and 18). Lane 19: negative control (yeast RNA). Lane 20: molecular weight marker (500, 400, 300, 200, 100 bp). Lane 21: 0.5 μ g total RNA of COS cells transfected with ER α plasmid was hybridized with the SR set. A combination of the unhybridized SR probes and the 18S probe are shown on the right side of the figure.

Figure 2 VDR, RAR and RXR mRNA expression levels in MCF-7 (lanes 1–6), T-47D (lanes 7–12) and ZR-75-1 cells (lanes 13–18) were determined using RPA. Control samples (no hormonal treatment) were run in lanes 1, 7 and 13. Cells were treated with 1 µg/ml insulin for 24 h (lanes 2, 8 and 14) or with 1 nmol/l estradiol for 24 h (lanes 3, 9 and 15) or with a combination of insulin and estradiol for 24 h (lanes 4, 10 and 16) or cells were grown in the presence of insulin and estradiol for 1 week (lanes 5, 11 and 17) and were treated for 24 h with 10 nmol/l R5020 (lanes 6, 12 and 18). Lane 19: negative control (yeast RNA). Lane 20: molecular weight marker (300, 200, 100 bp). Lane 21: COS cells transfected with either RARα or RARγ plasmid were hybridized separately with the VDR set and pooled together for gel analysis. The unhybridized VDR probes are shown on the right side of the figure.

Regulation of NR expression by estradiol

Regulation of NR mRNA expression was studied using cells treated for 24 h with insulin (1 µg/ml) or estradiol (1 nmol/l) or with a combination of both. Representative RPA gels are shown in Figs 1 and 2. A result was considered significant if two criteria were met. First, the hormonal treatment changed the expression level on average twofold or more when compared with control cells. Secondly, the results of three to five repeats gave P values <0.05 in the Mann–Whitney U test. Insulin alone did not significantly regulate the expression of any mRNAs tested. Since all estradiol-regulated receptors were also regulated by a combination treatment of estradiol and insulin, the results of only the latter are shown in Fig. 3A (SR set) and Fig. 4A (VDR set). Estradiol alone and the combination of estradiol and insulin increased the expression of PR and ERβ in all three cell lines. In addition, both treatments increased the expression of RARα mRNA in ZR-75-1 cells. The only differences between estradiol and the combination of estradiol and insulin treatments

Figure 3 Regulation of steroid receptor mRNA expression by insulin and estradiol or progestin R5020. MCF-7, T-47D and ZR-75-1 cells were treated with estradiol (E_2 ; 1 nmol/l) and insulin (1 µg/ml) for 24 h (A) or for 1 week (B). Cells grown in the presence of estradiol and insulin (1 week) were stimulated with R5020 (10 nmol/l) for 24 h (C). Values represent the change of expression levels (means $\pm$ S.D.) when hormone-treated samples were compared with controls. The experiment was repeated three to five times. The values of ERα are based on real-time RT-PCR analysis. * $P < 0.05$ in the Mann–Whitney U test; ^amean based on two values; ^bmean based on two values, the third experiment was up-regulated but the intensity of the control sample was below the detection limit.

Figure 4 Regulation of VDR, RAR γ and RAR α mRNA expression by insulin and estradiol or progestin R5020. MCF-7, T-47D and ZR-75-1 cells were treated with estradiol (E_2 ; 1 nmol/l) and insulin (1 μ g/ml) for 24 h (A) or for 1 week (B). Cells grown in the presence of estradiol and insulin (1 week) were stimulated with R5020 (10 nmol/l) for 24 h (C). The values represent the change of expression levels (means $\pm$ S.D.) when hormone-treated samples were compared with controls. The experiment was repeated three to five times. The values of VDR are based on real-time RT-PCR analysis. * $P < 0.05$ in the Mann–Whitney U test; ^amean based on two values.

were seen in T-47D cells. Estradiol and insulin together decreased the expression of AR and increased the expression of RAR α although estradiol alone could not induce significant changes. A probe for cyclin A was included in order to control the effects of hormonal treatments on cell proliferation. As expected, the expression of cyclin A was increased significantly in all cell lines after addition of estradiol, as well as after addition of both estradiol and insulin (data not shown).

Since the probe for ER α mRNA was shortest and contained less UTP than other probes in the SR set,

the signals obtained from ER α were weaker. Also the signals obtained from VDR expression in MCF-7 and ZR-75-1 cells were barely detectable due to the low expression level of VDR. In order to confirm the results obtained from the RPA analysis, the expression levels of ER α and VDR were also studied using quantitative real-time RT-PCR. The criteria for significant changes were the same as in the RPA analysis. The results from RT-PCR revealed that ER α expression was increased by estradiol alone and estradiol and insulin in T-47D cells and decreased in ZR-75-1 cells. A statistically significant trend towards decreased expression of ER α was observed in MCF-7 cells both after estradiol and after estradiol and insulin treatment; however, the result did not meet the criteria of the twofold change in the expression level. Treatment with either estradiol alone or a combination of estradiol and insulin for 24 h did not significantly affect the expression of VDR in any cell line analyzed.

Regulation of NR expression by long-term estradiol treatment

We also studied the long-term effects of estradiol and insulin on the expression of NRs. Expression levels of receptors in cells cultured for 1 week in the presence of estradiol (1 nmol/l) and insulin (1 μ g/ml) were compared with those of control cells grown for 1 week without estradiol and insulin. The results of the SR set are shown in Fig. 3B and the results of the VDR set in Fig. 4B. In all three cell lines, the expression of PR and ER β were increased, as they were already after 24 h stimulations. Long-term hormonal treatment decreased the expression of GR in MCF-7 cells. A similar trend was observed in ZR-75-1 and T-47D cells but these results were not statistically significant. Regulation of AR, RAR α , RAR γ , VDR and ER α mRNAs were cell line specific. Long-term hormonal treatment decreased the expression of AR in MCF-7 cells, whereas in ZR-75-1 cells the expression of RAR α and RAR γ was increased. Expression of ER α and VDR was studied using quantitative real-time RT-PCR. In MCF-7 and ZR-75-1 cells the expression of ER α was decreased and in T-47D cells increased by estradiol and insulin. Expression of VDR was only decreased in T-47D cells.

Regulation of NR expression by progestin

Regulation of NR expression by the progestin R5020 was studied using cells grown for 1 week in the presence of insulin (1 μ g/ml) and estradiol (1 nmol/l). The samples were collected 24 h after the addition of 10 nmol/l R5020. The results of the SR set are shown in Fig. 3C and the results of the VDR set in Fig. 4C. Expression of PR and ER β was decreased in all cell lines. In addition, R5020 treatment decreased the expression of RAR α in T-47D cells. Progestin augmented the expression level of VDR in T-47D cells.

Since the other cell lines expressed VDR only at a low level, we also analyzed the expression levels of VDR by quantitative real-time RT-PCR. Results obtained from RT-PCR suggested that VDR expression was increased by progestin in MCF-7 and T-47D cells. In ZR-75-1 cells the increase of VDR expression was consistent but did not fulfil the criteria of significant regulation, since the mean increase was only 1.57 (s.d. ± 0.08). Expression of ER α was also studied using RT-PCR. R5020 treatment decreased the expression of ER α in T-47D and ZR-75-1 cells.

Expression of cofactors

The expression of different cofactors was characterized using RPA (Fig. 5A). In MCF-7 cells the gene coding for AIB1 is amplified and the cells expressed mRNA abundantly as reported earlier (6, 52). Also T-47D and ZR-75-1 cells expressed AIB1 mRNA, but at lower levels. N-CoR, pCAF and CBP were expressed in all cell lines studied. Coactivators p300 and TIF2 and corepressor SMRT were also expressed in all cell lines but the expression levels were too low to be quantitated reliably. Expressions of SRC-1a and SRC-1e mRNAs

were not detected with the RPA analysis from breast cancer cells in this study. However, Kalkhoven *et al.* (53) have shown that SRC-1a and SRC-1e mRNAs are expressed in MCF-7, T-47D and ZR-75-1 cells. Fig. 5B shows RPA from HL-60 cells, demonstrating that all the probes of the cofactor set identified corresponding mRNA.

Regulation of cofactor expression

The expression profile of cofactors AIB1, N-CoR, CBP and pCAF did not change in the short-term (24 h) treatment of cells with insulin, estradiol or with a combination of both estradiol and insulin (Fig. 6A). Neither did it change after long-term (1 week) treatment with insulin and estradiol (Fig. 6B). In addition, regulation of cofactor expression was not observed when cells were first grown for 1 week in the presence of insulin and estradiol and were then treated with R5020 for 24 h (Fig. 6C). Because the expression levels of SMRT, TIF2 and p300 in all cell lines were too low to be quantified accurately, we could not conclude whether hormonal treatments regulated the expression levels of these genes.

Figure 5 (A) Cofactor mRNA expression levels in MCF-7 (lanes 2–5), T-47D (lanes 6–11) and ZR-75-1 cells (lanes 12–17) were determined using RPA. Control samples (no hormonal treatment) were run in lanes 2, 6 and 12. Cells were treated with 1 μ g/ml insulin for 24 h (lanes 3, 7 and 13) or with 1 nmol/l estradiol for 24 h (lanes 4, 8 and 14) or with a combination of insulin and estradiol for 24 h (lanes 5, 9 and 15) or cells were grown in the presence of insulin and estradiol for 1 week (lanes 10 and 16) and were treated for 24 h with 10 nmol/l R5020 (lanes 11 and 17). Lane 1: negative control (yeast RNA). The unhybridized cofactor probes are shown on the right. (B) Cofactor mRNA expression in HL-60 cells treated with 8CTP-cAMP (200 mmol/l) and SR11237 (1 mmol/l) for 48 h (lane 1) and negative control (yeast RNA, lane 2). The unhybridized cofactor probes are shown on the left side of the figure. The SRC-1 probe hybridizes to both SRC-1e (length of the protected fragment is 160 bp) and SRC-1a (145 bp).

Figure 6 Regulation of cofactor mRNA expression by insulin and estradiol or progestin R5020. MCF-7, T-47D and ZR-75-1 cells were treated with estradiol (1 nmol/l) and insulin (1 µg/ml) for 24 h (A) or for 1 week (B). Cells grown in the presence of estradiol and insulin (1 week) were stimulated with R5020 (10 nmol/l) for 24 h (C). The values represent the change of expression levels (means ± s.d.) when hormone-treated samples were compared with controls. The experiment was repeated three to five times. * $P < 0.05$ in Mann–Whitney U test; ^amean based on two values.

Discussion

NRs play an important role in the regulation of cell growth, differentiation and apoptosis. Thus they have wide potential as pharmaceutical targets. In endocrine therapy, both natural and synthetic ligands have been used for decades in the treatment of normal and malignant tissues. The current development of novel NR ligands focuses on tissue-selective receptor modulators, which have different functions depending on the target organ. The existence of these tissue-selective receptor modulators demonstrates that the expression of

particular NR cannot sufficiently determine the function that its ligand has in cells. The mechanisms behind the tissue specificity are not entirely known, but the expression and post-transcriptional regulation of cofactors are suggested to have a substantial role (54). For example, the tissue-selective effects of tamoxifen were recently shown to be due to cell type and promoter-dependent recruitment of cofactors (32).

Here we have characterized three breast cancer cell lines for their NR and cofactor profile. The results showed that different breast cancer cell lines had considerably different expression profiles. For example, T-47D cells expressed ER β , PR and VDR most abundantly, whereas, ZR-75-1 cells expressed ER α , AR and GR most abundantly. The expression of VDR was lowest in MCF-7 cells. Among the cofactors studied the expression of AIB1 varied most, since in MCF-7 cells the gene coding for AIB1 is amplified and these cells express AIB1 mRNA at very high levels (6). The other cell lines expressed AIB1 as well, but at lower levels, which was consistent with the study by Thenot *et al.* (52). However, Anzick *et al.* (6) showed that AIB1 is also amplified and overexpressed in ZR-75-1 cells. These different results might be due to the different cell clones used. Coactivators pCAF and CBP and corepressor N-CoR were detected in all cell lines. Expression of cofactors p300 and SMRT were also detected; however, the levels were too low to be quantitated reliably. As reported here, there are differences in the expression levels of NRs and cofactors in different cell lines and thus these cell lines have different determinants for the effects of ligands. Results suggest that breast cancers from different patients as well as from different cancer cell clones in one particular tumor can vary considerably in regard to the NR and cofactor expression profile. This evidence may have an implication in the development of novel ligands for HRT and cancer treatment as well as for the molecular diagnostics of breast cancers.

In this study, a cell line-specific regulation of several NRs by estradiol or progestin was observed. The amount of RAR α mRNA was up-regulated in T-47D and ZR-75-1 cells after 24 h of estradiol and insulin stimulation. In ZR-75-1 cells the increased RAR α expression was also observed in cells treated only with estradiol for 24 h and in cells grown for 1 week in the presence of estradiol and insulin. We could not detect consistent regulation of RAR α in MCF-7 cells although van der Leede *et al.* (55) have already reported increased expression of RAR α after estradiol treatment. A novel finding of our study was the increased expression of RAR γ in ZR-75-1 cells after long-term treatment with estradiol and insulin. Increased expression was unique to this cell line only. Previously, it has been suggested that estradiol does not regulate RAR γ expression in T-47D cells (56). Long-term estradiol and insulin treatment decreased the expression of VDR in T-47D cells. This is in contrast

to a previous study in which high (100 nmol/l) estradiol concentration induced a twofold increase of vitamin D binding in MCF-7 cells (57). The expression of AR was decreased when T-47D cells were treated for 24 h and MCF-7 cells were treated for 1 week with estradiol and insulin. A reduced binding of dihydrotestosterone has been shown, maximally 6 days after estradiol treatment in MCF-7 cells (58). However, Hall *et al.* (59) did not find regulation of AR levels in T-47D or in MCF-7 cells by using Northern blot even 3 days after estradiol treatment. Long-term treatment with estradiol and insulin also reduced the expression level of GR in MCF-7 cells. This is consistent with a study showing decreased expression of GR mRNA in MCF-7 cells after 48 h estradiol treatment but not after shorter treatments (60). A similar trend towards decreased expression of GR was also observed in T-47D and ZR-75-1 cells.

Estradiol had controversial effects on ER α expression; it increased the expression in T-47D cells and a trend towards decreased expression was seen in MCF-7 cells, as described previously (37–39). The expression of ER α was decreased in ZR-75-1 cells, which is in contrast to the study by Clayton *et al.* (38) in which estradiol increased the ER α mRNA expression in ZR-75 cells. Different ZR-75 cell clones used might explain the difference in the results. Expression of ER β and PR were induced by estradiol in all cell lines studied. The inductions were seen after 24 h treatment as well as after 1 week of treatment. This is the first study describing the fact that ER β is regulated in a similar manner in MCF-7 and ZR-75-1 cells, as described earlier for T-47D cells (40).

Cell line-specific regulation was seen after 24 h of R5020 treatment. Progestin decreased the expression of RAR α in T-47D cells, as described earlier (61). On the other hand, progestin stimulated the expression of VDR in MCF-7 and T-47D cells. Interestingly, ligands of both RAR α and VDR have been shown to promote cell differentiation and inhibit cell proliferation; thus our results suggest that progesterone treatment might modulate the effect of retinoids and vitamin D in the mammary gland. The expression of ER α was decreased in T-47D and ZR-75-1 cells by progestin. However, the level of ER α in MCF-7 cells was not significantly decreased. Savoldi *et al.* (62) suggested that this might be due to lower PR levels in MCF-7 cells compared with other cell lines. A novel finding was that the expression of ER β was also reduced in MCF-7 and ZR-75-1 cells by progestin as described earlier for T-47D cells (43). Thus, treatment of breast cancer by progestins might decrease the expression of both ER α and ER β , decreasing the growth-stimulatory responses of endogenous estrogens.

We did not detect a significant regulation of expression of corepressors N-CoR and SMRT or coactivators AIB1, pCAF and CBP after estradiol treatment. This is in agreement with other studies in which

estradiol treatment did not regulate the expression of TIF1 α , AIB1, SMRT or SRC-1 in MCF-7 cells (52) or SRC-1, glucocorticoid receptor-interacting protein-1, AIB1, receptor-interacting protein-140 or p300 in the rat uterus (63). Yet there is evidence that some cofactors are regulated by estradiol. In MCF-7 cells RIP140 mRNA expression was increased by estradiol (52). The regulation of SRC-1 and SMRT by estradiol has also been shown in the rat pituitary (64). In addition, other NR ligands have been shown to regulate cofactors. For example, all-*trans* retinoic acid treatment increased AIB1 expression in HL-60 cells (65) and SMRT expression in neuroblastoma cell lines (66). Thyroid hormone treatment slightly increased SRC-1 expression in the rat pituitary (64). However, whether progestins regulate cofactor expression has not been studied earlier. Our results imply that progestin does not regulate the expression level of AIB1, pCAF, CBP and N-CoR mRNAs. Possibly the activity of cofactors is regulated through post-transcriptional modifications such as phosphorylation, methylation, acetylation and ubiquitination rather than by hormonal regulation of expression levels (54).

The results presented here show that the expression profile of NRs is cell clone dependent and steroid regulated. An important finding here is that the cofactors have a breast cancer cell type-specific expression pattern but their expression levels are not hormonally regulated. A cell type-specific expression pattern of the NR and cofactor expression implies that different clones of the same breast tumor might have different NR and cofactor expression profiles, which brings an additional challenge in the development of novel selective receptor modulators.

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